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Anticarcinogenic Activities of Organic Isothiocyanates: Chemistry and Mechanisms<sup>1</sup>

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## Abstract

Organic isothiocyanates block the production of tumors induced in rodents by diverse carcinogens (polycyclic aromatic hydrocarbons, azo dyes, ethionine, *N*-2-fluorenylacetylamide, and nitrosamines). Protection is afforded by  $\alpha$ -naphthyl-,  $\beta$ -naphthyl-, phenyl-, benzyl-, phenethyl-, and other arylalkyl isothiocyanates against tumor development in liver, lung, mammary gland, forestomach, and esophagus. Many isothiocyanates and their glucosinolate precursors ( $\beta$ -thioglucoside, *N*-hydroxysulfate) occur naturally and sometimes abundantly in plants consumed by humans, e.g., cruciferous vegetables. Nevertheless, the possible contributions of isothiocyanates and glucosinolates to the well recognized protective effects against cancer of high consumptions of vegetables are unclear. The anticarcinogenic effects of isothiocyanates appear to be mediated by tandem and cooperating mechanisms: (a) suppression of carcinogen activation by cytochromes P-450, probably by a combination of down-regulation of enzyme levels and direct inhibition of their catalytic activities, which thereby lower the levels of ultimate carcinogens formed; and (b) induction of Phase 2 enzymes such as glutathione transferases and NAD(P)H: quinone reductase, which detoxify any residual electrophilic metabolites generated by Phase 1 enzymes and thereby destroy their ability to damage DNA. Since isothiocyanates block carcinogenesis by dual mechanisms and are already present in substantial quantities in human diets, these agents are ideal candidates for the development of effective chemoprotection of humans against cancer.

## Introduction

Organic isothiocyanates ( $R-N=C=S$ ), also known as mustard oils, are widely distributed in plants, many of which are consumed by humans. They are responsible for the pungent and acrid flavor and odor of condiments such as mustard and horseradish and the familiar biting taste that develops when some cruciferous vegetables are eaten. The designation "mustard oil" originates from the flavor of mustard seeds which is largely due to the presence of abundant quantities of allyl isothiocyanate ( $CH_2=CH-CH_2-NCS$ ). In plants, isothiocyanates are invariably accompanied by usually much larger quantities of their cognate glucosinolates ( $\beta$ -thioglucoside, *N*-hydroxysulfate). In addition to their characteristic flavors and odors, isothiocyanates have a variety of other pharmacological and toxic activities. These include: goitrogenic activity; antibacterial, antifungal, and antiprotozoal actions; the ability to attract or repel insects; cytotoxicity; the induction of chromosome abnormalities and neoplasia; and the blocking of chemical carcinogenesis. The interesting early history of the chemistry and biology of these compounds has been reviewed by Challenger (1). Several more recent encyclopedic and scholarly reviews of the chemistry, distribution in plants, biosynthesis, and biological properties of glucosinolates and isothiocyanates are available (2-4).

Isothiocyanates arise in plants as a result of enzymatic cleavage of glucosinolates by myrosinase (thioglucoside glucosylhydrolase, EC 3.2.3.1), which is released when plant cells are injured. Myrosinase promotes the hydrolysis of glucosinolates and intramolecular (Lossen) rearrangement of intermediates to yield isothiocyanates, hydrogen sulfate, and glucose as the major products (Fig. 1).

As shown in Fig. 1, a number of other products may also arise from

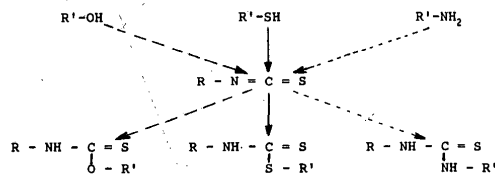
the hydrolysis of glucosinolates by myrosinase. The quantities and nature of these products are variable and appear to be controlled by the chemical nature of the glucosinolate, the pH, and the origin and multiplicity of the myrosinases. A full discussion of the formation of these products is provided by Fenwick *et al.* (2).

Our interest in isothiocyanates stemmed from the observations that several members of this family of substances could block the toxic and neoplastic effects of a wide variety of chemical carcinogens (6). Furthermore, many isothiocyanates are monofunctional inducers of Phase 2 enzymes (7, 8), a property that is associated with protection against chemical carcinogenesis (9). Recently, a very potent isothiocyanate inducer (sulforaphane) has been isolated from broccoli (10). These observations point to the potential importance of isothiocyanates as chemoprotectors against cancer in humans.

This review focuses on the metabolism of isothiocyanates and on the mechanisms of their anticarcinogenic effects. We discuss only experiments in which pure isothiocyanates have been used and omit corroborating information obtained with plants and their extracts, because of the presence of other potentially confounding substances.

## Chemical Properties and Metabolism of Isothiocyanates

**Chemical Reactivity and Spectroscopic Properties.** The highly electrophilic central carbon atom of the  $-N=C=S$  group reacts rapidly, and under mild conditions with oxygen-, sulfur-, or nitrogen-centered nucleophiles to give rise to carbamates, thiocarbamates, or



thiourea derivatives, respectively.

These reaction products have been useful for the spectroscopic identification and characterization of isolated isothiocyanates. The  $-NCS$  group of isothiocyanates absorbs UV light with low intensity near 240 nm ( $\epsilon_m$  about  $1000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Reactions of isothiocyanates with monothiolates at pH 7-9 yield thiocarbamates (carbamate thioesters) that show markedly enhanced UV absorption intensity ( $\epsilon_m$   $10,000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and a characteristic maximum at 270 nm with a broad shoulder near 250 nm (11-13). Zhang *et al.* (13) have reported recently that isothiocyanates react with vicinal dithiols, such as 2,3-dimercaptopropanol or ethane-1,2-dithiol, to give rise initially to the expected monothiocarbamates. However, upon further incubation these primary products undergo attack by the free thiol group on the electrophilic carbon, resulting in a cyclization reaction with release of the free amine (13, 14). Thus the reaction of any  $R-NCS$  with ethane-1,2-dithiol, 2,3-dimercaptopropanol, and 1,2-benzenedithiol gives rise to ethylene trithiocarbonate (1,3-dithiolane-2-thione,  $\lambda_{max}$  316 nm;  $\epsilon_m$   $16,500 \text{ M}^{-1} \text{ cm}^{-1}$ ), 4-hydroxymethyl-1,3-dithiolane-2-thione ( $\lambda_{max}$  316 nm;  $\epsilon_m$   $16,400 \text{ M}^{-1} \text{ cm}^{-1}$ ), and 1,3-benzodithiole-2-thione ( $\lambda_{max}$  363 nm;  $\epsilon_m$   $22,500 \text{ M}^{-1} \text{ cm}^{-1}$ ), respectively. Based on the

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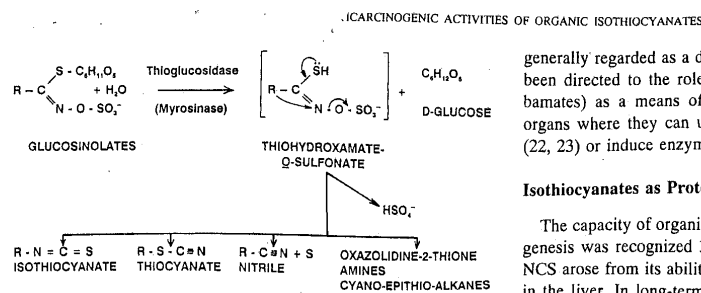


Fig. 1. Hydrolysis of glucosinolates by myrosinase and formation of isothiocyanates and other products [from Pessina *et al.* (5)].

favorable spectroscopic properties of 1,3-benzodithiole-2-thione, a general method for the sensitive, specific, and quantitative analysis of isothiocyanates was developed (13).

**Metabolism of Isothiocyanates *in Vivo*.** Whereas the nonenzymatic reaction of GSH<sup>2</sup> with benzyl-NCS is rapid, this reaction is also catalyzed by rat liver cytosols, presumably promoted by the glutathione transferases present in these preparations (15). The principal metabolic products of isothiocyanates administered *p.o.* to mammals are the corresponding thiocarbamates. When six male volunteers were fed about 100  $\mu\text{mol}$  of benzyl-NCS, 53.7% of the dose administered was excreted in the urine as *N*-acetyl-S-(*N*-benzylthiocarbamoyl)-L-cysteine, a mercapturic acid, in the first 12 h. No other metabolites were detected, and the excretion appeared to be complete in this period (16). Similar results were observed in two men and two women who were fed watercress, a rich source of phenethyl-NCS and its glucosinolate (gluconasturtiin), which is hydrolyzed by the myrosinase present in this plant (17). When 30- or 57-g portions of fresh watercress (containing 0.72 mg of gluconasturtiin/g) were consumed by each individual, 30 to 67% of the phenethyl-NCS was excreted as *N*-acetylcysteine derivative in the urine in 24 h, and the majority of this conjugate was recovered within the initial 8 h (17). This experiment does not provide unequivocal evidence for the hydrolysis of the glucosinolate to isothiocyanate in the body, since no information on the content of free phenethyl-NCS in the watercress was provided. Furthermore, it is not clear whether breakdown of the glucosinolate to isothiocyanate resulted from the myrosinase activity of the watercress or from enzymes present in the test subjects (either in the host tissues or the microbial flora of the gastrointestinal tract). We are not aware of any experimental information on the ability of human subjects to hydrolyze glucosinolates to isothiocyanates.

Rats, mice, guinea pigs, and rabbits likewise converted isothiocyanates efficiently to *N*-acetylcysteine conjugates, but other metabolites, including cyclic mercaptopyruvate derivatives (not detected in humans) were also formed (17-20). However, when phenyl-,  $\alpha$ -naphthyl-,  $\beta$ -naphthyl, or *tert*-butyl isothiocyanate were given to rats, no cysteine conjugates were detected (15, 16, 21).

The conversion of isothiocyanates to their *N*-acetylcysteine derivatives (mercapturic acids) proceeds by the conventional route of conjugation with glutathione presumably promoted by glutathione transferases. The resulting conjugates are then hydrolyzed to the cysteine derivatives and acetylated. Although this pathway has been

generally regarded as a detoxication mechanism, recent attention has been directed to the role of GSH and cysteine conjugates (thiocarbamates) as a means of transporting isothiocyanates to peripheral organs where they can undergo cleavage and contribute to toxicity (22, 23) or induce enzymes that protect against toxicity (see below).

#### Isothiocyanates as Protectors against Chemical Carcinogenesis

The capacity of organic isothiocyanates to block chemical carcinogenesis was recognized 30 years ago (24, 25). Interest in naphthyl-NCS arose from its ability to produce profound proliferative damage in the liver. In long-term feeding experiments  $\alpha$ -naphthyl-NCS significantly reduced (in a dose-dependent manner) the formation of liver tumors by 3'-methyl-4-dimethylaminoazobenzene, ethionine, and *N*-2-fluorenylacetylamide in male Wistar rats. Furthermore, animals fed  $\alpha$ -naphthyl-NCS did not develop ear duct carcinomas and leukemia, unlike rats receiving the *N*-2-fluorenylacetylamide alone (24, 25). Lacassagne *et al.* (26) confirmed and extended these findings by demonstrating that not only dietary  $\alpha$ -naphthyl-NCS but also  $\beta$ -naphthyl-NCS (which does not cause marked proliferative changes in the liver) blocked hepatic tumor formation in rats fed 4-dimethylaminoazobenzene. Both isothiocyanates produced profound effects on hepatic enzymes that metabolize xenobiotics (27, 28).

These findings laid the groundwork for many subsequent studies on the tumor blocking activities of isothiocyanates, which were administered usually for only short time periods (Table 1).

**Mammary Tumors.** In the single dose 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary tumor model in female Sprague-Dawley rats, single doses of phenyl-NCS, phenethyl-NCS, and benzyl-NCS markedly reduced the incidence and multiplicity of mammary tumors. In a detailed experiment Wattenberg (29) showed that the timing of administration of the anticarcinogen with respect to the DMBA was very important. Thus, when benzyl-NCS was administered by gavage 2 h prior to the single dose of carcinogen, the average number of rats bearing tumors was reduced from 77% to 8%, and the number of tumors per animal dropped from 1.6 to 0.08. Administration of the isothiocyanate 4 h prior to the carcinogen was somewhat less effective; but if the isothiocyanate was given 24 h before or 4 h after the DMBA, the tumor blocking effect was largely abolished. In other experiments with the same rat tumor model, Wattenberg (30) observed that dietary benzyl isothiocyanate fed for the entire experimental period, beginning 1 week after the DMBA dose, was also highly effective in reducing mammary tumor incidence and multiplicity.

**Forestomach and Lung Tumors.** Administration of benzyl-NCS (1 mg) to female A/J mice by gavage 15 min prior to each exposure to dimethylnitrosamine (20 mg/kg; once weekly for 8 weeks) reduced forestomach tumor incidence and multiplicity but had no effect on pulmonary adenoma formation. In contrast, administration of benzyl-NCS (1 or 2.5 mg, 15 min before carcinogen) reduced, in a dose-dependent manner, both pulmonary adenomas and forestomach tumors evoked by benzo(a)pyrene (3 doses of 2 mg each, given *p.o.* at 2-week intervals) (31). Benzyl-NCS also suppressed the formation of forestomach tumors in ICR/Ha mice (38).

The effects of isothiocyanates on lung tumors resulting from administration of the potent tobacco-derived nitrosamine carcinogen NNK have been studied intensively and reviewed recently by Chung (39). In male F344 rats, phenethyl-NCS was fed for 1 week prior to and during the course of treatment with NNK (1.76 mg/kg *s.c.*, 3 times weekly for 21 weeks). At the termination of the experiment (104 weeks), the pulmonary tumor incidence (adenomas and carcinomas) was reduced from 80% to 43% by the isothiocyanate treatment, which also inhibited the methylation and pyridyloxobutylolation of lung DNA (32).

In shorter term experiments, female A/J mice received 5  $\mu\text{mol}$  of various phenyl-(CH<sub>2</sub>)<sub>n</sub>-NCS (*n* = 0-6) daily by gavage for 4 days.

<sup>2</sup> The abbreviations and definitions used are: GSH, glutathione; OR, quinone reductase [NAD(P)H:quinone acceptor oxidoreductase, EC 1.6.99.2]; GST, glutathione transferases (EC 2.5.1.18); Phase 1 enzymes (principally cytochromes P-450) functionalize xenobiotics largely by oxidative or reductive reactions; Phase 2 enzymes conjugate functionalized compounds with endogenous ligands (e.g., GSH, glucuronic acid). OR is classified as a Phase 2 enzyme. Monofunctional inducers elevate Phase 2 enzymes without intervention of the Ah (Aryl hydrocarbon) receptor and do not significantly affect Phase 1 enzyme activities; DMBA, 9,10-dimethyl-1,2-benzanthracene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; i.g., intragastrically.

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Table 1 Protection against chemical carcinogenesis by aryl and arylalkyl isothiocyanates in rats and mice

Carcinogen	Species (sex/strain)	Tumor target organ	Protective isothiocyanate	Ref.
3'-Me-DAB <sup>a</sup>	Rat (♂, Wistar)	Liver	$\alpha$ -Naphthyl-	Sasaki (25)
Ethionine	Rat (♂, S-D)	Liver	$\alpha$ -Naphthyl-	Sidransky <i>et al.</i> (24)
AAF	Rat (♂, Wistar)	Liver, leukemia, ear duct	$\alpha$ -Naphthyl-	Sidransky <i>et al.</i> (24)
DAB	Rat (♂, Wistar)	Liver	$\alpha$ - and $\beta$ -naphthyl-	Lacassagne <i>et al.</i> (26)
DMBA	Rat (♀, S-D)	Mammary gland	Phenyl-, benzyl-, phenethyl-	Wattenberg (29, 30)
DMBA	Mouse (♀, ICR/Ha)	Lung, forestomach	Benzyl-, phenethyl-	Wattenberg (29)
BP	Mouse (♀, ICR/Ha)	Forestomach	Benzyl-	Wattenberg (29)
DEN	Mouse (♀, A/J)	Forestomach	Benzyl-	Wattenberg (31)
BP	Mouse (♀, A/J)	Lung, forestomach	Benzyl-	Wattenberg (31)
NNK	Rat (♂, F344)	Lung	Phenethyl-	Morse <i>et al.</i> (32)
NNK	Mouse (♀, A/J)	Lung	Phenyl- (CH <sub>2</sub> ) <sub>n</sub> (n = 0-6)	Morse <i>et al.</i> (33-35)
NBMA	Rat (♂, F344)	Esophagus	Phenethyl-	Stoner <i>et al.</i> (36, 37)

<sup>a</sup> 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene; AAF, *N*-2-fluorenylacetylamide; BP, benzo(a)pyrene; DEN, diethylnitrosamine; NBMA, *N*-nitrosobenzylmethylamine.

Two h after the final treatment with isothiocyanates, a single dose of NNK (10  $\mu$ mol by i.p. injection) was administered and pulmonary tumors were quantitated 16 weeks later. Phenyl-NCS and benzyl-NCS were inactive, but phenethyl-NCS ( $n = 2$ ) and isothiocyanates with  $n = 3-6$  produced marked reductions in lung tumor formation; the inhibition was progressively more pronounced as the methylene chain was lengthened (33, 34). Thus phenylhexyl-NCS (5  $\mu$ mol daily for 4 days) reduced pulmonary tumor multiplicity from 7.9 to essentially zero. DNA methylation, as measured by *O*<sup>6</sup>-methylguanine formation, was likewise blocked by these arylalkyl isothiocyanates, again more markedly as the methylene chain of the isothiocyanates was made longer (34).

If the administration of benzyl-NCS or phenethyl-NCS (1-3  $\mu$ mol/g of diet) was delayed for 1 week after treatment with NNK and then continued to the end of the experiment at 16 weeks, no effect on pulmonary tumor formation was observed at nontoxic doses of the isothiocyanates (35).

**Tumors of the Esophagus.** Stoner *et al.* (36) have reviewed their studies on the inhibition by phenethyl-NCS of esophageal tumor production in the rat by the asymmetrical nitrosamine, *N*-nitrosobenzylmethylamine. The importance of these studies derives from the belief that *N*-nitroso compounds and their precursors are probably causative factors for esophageal cancers in some high incidence regions (37). Male F344 rats treated with *N*-nitrosobenzylmethylamine, (0.5 mg/kg s.c. once per week for 15 weeks) developed 100% esophageal tumors at the end of the 25-week assay period, and the tumor multiplicity was 11.5/animal. In experimental groups also fed phenethyl-NCS (3  $\mu$ mol/g of diet), tumor incidence was only 13% and average tumor multiplicity was negligible ( $0.1 \pm 0.3$ /animal). At a higher dose of phenethyl-NCS (6  $\mu$ mol/g of diet) no tumors were observed (37). Phenethyl-NCS treatment blocked the formation of both preneoplastic and neoplastic lesions in the esophagus; even at the lower dose of the isothiocyanate, there was complete inhibition of the appearance of the more advanced (papilloma and carcinoma) lesions (37).

Parallel measurements of the effects of phenethyl-NCS on the metabolism of [<sup>3</sup>H]nitrosobenzylmethylamine in rat esophageal explants demonstrated concentration-dependent inhibition of both metabolism of the carcinogen and methylation of DNA, as indicated by decreased formation of *N*<sup>7</sup>-methylguanine and *O*<sup>6</sup>-methylguanine.

**Tumor-blocking Effects of Glucosinolates.** A few experiments have examined the chemoprotective effects of glucosinolates, which are often present in plants in much higher concentrations than their isothio-

cyanate hydrolysis products. The most clear-cut results have been obtained with the DMBA-induced mammary tumors of the Sprague-Dawley rat. Administration of large single doses of glucobrassicin (indolylmethyl glucosinolate) or of glucotropaeolin (benzyl glucosinolate) 4 h prior to the carcinogen substantially reduced both the incidence (from 75% to 25-38%) and the multiplicity (from 1.25 to 0.50-0.69 tumor/rat) of mammary tumors. Administration of these glucosinolates or of glucosinalbin (4-hydroxybenzyl glucosinolate) produced some reduction in the multiplicity, but usually not the incidence, of forestomach tumors and pulmonary adenomas of mice treated with benzo(a)pyrene (40). The interpretation of these experiments is complicated. It is not known to what extent these glucosinolates are degraded in the rodent bodies or to what extent the glucosinolates or their degradation products are responsible for the tumor-blocking effects. If the glucosinolates are hydrolyzed, it is not known whether endogenous or microbial enzymes are responsible and what products are formed. These issues are especially complicated for glucobrassicin, since three known degradation products of this glucosinolate (indole-3-acetonitrile, 3,3'-diindolylmethane, and indole-3-carbinol) are anticarcinogens and inducers of cytochrome P-450 (41, 42). A further potentially confounding effect is the conversion of indole-3-carbinol under mildly acid conditions (such as prevail in the stomach) to cyclic derivatives that bind at very low concentrations to the Ah receptor and thereby become very potent inducers of cytochrome P450IA1 (43).

#### Mechanisms: Effects of Isothiocyanates on Carcinogen Metabolism

Understanding of the mechanisms of the chemoprotective effects of isothiocyanates is of great importance not only because these substances block the formation of a wide variety of carcinogen-induced tumors in rodents, but also because isothiocyanates and their glucosinolate precursors are widespread in human dietary plants and are consumed in substantial quantities. To what extent these substances contribute to the well-recognized protective effects of vegetables against cancer is unclear (44). The only plausible mechanisms proposed for the anticarcinogenic effects of isothiocyanates implicate modulation of carcinogen metabolism, both depression of activation of carcinogens and acceleration of their disposal. Evidence for these conclusions is based on measurements of: (a) carcinogen-DNA adduct formation and the accompanying nucleotide modifications; (b)

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Table 2 Induction of Phase 2 enzymes by isothiocyanates in rodent tissues

Enzyme	R-NCS	Species (sex/strain)	Tissue	Ratio of enzyme specific activities (treated/control)	Ref.
GST	Benzyl-NCS	Mouse (female; ICR/Ha)	Esophagus	2.08-2.59	Sparnins <i>et al.</i> (38, 49, 50)
			Forestomach	2.46	
			Liver	3.20	
			Small bowel	4.38-9.36	
GST	Benzyl-NCS	Mouse (female; ICR/Ha)	Forestomach	3.43-4.29	Benson and Barretto (51) Benson <i>et al.</i> (52)
			Liver	3.0-3.5	
			Lung	1.63-1.73	
			Kidney	1.35-1.37	
			Small bowel	5.27-8.31	
			Colon	1.25	
GST	Allyl-NCS	Rat (male; F344)	Liver	1.26-2.25	Bogaards <i>et al.</i> (53)
GST	Phenethyl-NCS	Rat (male; F344)	Small bowel	3.07-3.93	Guo <i>et al.</i> (47)
			Liver	1.3	
GST	Phenylbutyl-NCS Phenylhexyl-NCS	Rat (male; F344)	Liver	1.35	Guo <i>et al.</i> (57)
			Liver	1.37	
GST	Sulforaphane Erucin Erysolin	Mouse (female; CD-1)	Forestomach	1.08-3.00	Zhang <i>et al.</i> (10)
			Glandular stomach		
			Liver		
			Small bowel Colon		
QR	Benzyl-NCS	Mouse (female; CD-1)	Forestomach	2.57	Benson and Barretto (51) Benson <i>et al.</i> (52)
			Liver	2.05	
			Lung	2.82	
			Kidney	2.27	
			Small bowel	4.78	
			Colon	1.80	
			Bladder	2.41	
QR	Phenethyl-NCS	Rat (male; F344)	Liver	5.1	Guo <i>et al.</i> (47)
			Lung	~1	
			Nasal mucosa	~1	
QR	Phenylbutyl-NCS Phenylhexyl-NCS	Rat (male; F344)	Liver	1.44	Guo <i>et al.</i> (57)
			Liver	1.63	
QR	$\alpha$ -Naphthyl-NCS $\beta$ -Naphthyl-NCS	Rat (male; F344)	Liver	2.8-4.4	Leonard <i>et al.</i> (27, 28)
			Liver	2.23	
QR	Sulforaphane Erucin Erysolin	Mouse (female; CD-1)	Forestomach	1.05-3.10	Zhang <i>et al.</i> (10)
			Glandular stomach		
			Liver		
			Small bowel Colon		

rates of activation of carcinogens and levels of Phase 1 enzymes; and (c) activities of Phase 2 enzymes and levels of GSH.

**Reactions of Carcinogens with DNA.** There is a striking parallel between the inhibitory effects of various arylalkyl isothiocyanates on lung tumor formation by NNK, the potent carcinogenic nitrosamine of tobacco, and the ability of these isothiocyanates to block *O*<sup>6</sup>-methylguanine formation in lungs of rats and mice. The very systematic studies by Chung, Hecht and their colleagues should be consulted for details [Refs. 32, 39, and 45; reviewed by Chung (46)]. These workers and others (46) have also studied the metabolic activation of NNK by lung tissue and microsomes and have concluded that the chemoprotective isothiocyanates act principally on the enzymes involved in the metabolic activation of NNK. By the use of inhibitory antibodies specific for cytochromes P-450, Guo *et al.* (46) identified cytochromes P450IIB1 and 2 but not P450IA1 or P450IIE1 as important participants in the activation process.

Similar experiments by Stoner *et al.* (36, 37) have established that the inhibitory effects of phenethyl-NCS on the production of esoph-

ageal tumors by *N*-nitrosobenzylmethylamine in rats also paralleled inhibition of the binding of the carcinogen to DNA and the formation of *N*<sup>7</sup>-methylguanine and *O*<sup>6</sup>-methylguanine.

**Regulation of Phase 1 Enzymes by Isothiocyanates.** Administration of isothiocyanates to rodents produced either increases or decreases of microsomal cytochrome P-450 content and the activities of several cytochrome P-450-dependent monooxygenases. The effects appear to depend on experimental conditions: the nature of the isothiocyanate; the treatment regimen; the target tissue examined; and the specific monooxygenase measured.

The most dramatic increases have been reported with indole-3-carbinol and 3,3'-diindolylmethane and to a lesser degree with indole-3-acetonitrile. These indoles are hydrolytic rearrangement products of glucobrassicin (indolylmethyl glucosinolate). Single i.g. administrations of 10-100  $\mu$ mol of these indoles to female Sprague-Dawley rats raised the aryl hydrocarbon hydroxylase activities of homogenates of liver and small intestine as much as 25-fold. Clearly these effects cannot be directly attributed to the actions of isothiocyanates (41). As already mentioned,



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recent experiments (43) have shown that indole-3-carbinol is readily converted both *in vitro* and *in vivo*, under mildly acid conditions to polymeric indoles that bind with very high affinity to the Ah receptor, and thereby enhance transcription of cytochrome P450A1.

Single i.g. doses (100  $\mu$ mol) of phenethyl-NCS to male F344 rats caused marked increases of liver microsomal pentoxyresorufin *O*-dealkylase activity (10-fold) and the content of cytochrome P450IIB1 protein (6.6-fold) (47). In contrast, cytochrome P-450 content, ethoxyresorufin *O*-dealkylase, and erythromycin *N*-demethylase activities all fell moderately during the first 12–18 h and then recovered by the end of the experiment at 48 h (47). Although these effects appear to be somewhat contradictory, the above-described treatment with phenethyl-NCS depressed the formation of reactive metabolites of NNK by lung (and to a lesser degree by liver) microsomes. The principal effect of phenethyl-NCS appears to be depression of the  $\alpha$ -hydroxylation of NNK which is critical for DNA adduct formation. Furthermore, the direct addition of a number of isothiocyanates to microsomes of rat and mouse lungs and rat nasal mucosa potently depressed the metabolic activation reactions of NNK. Thus, 50% inhibition was achieved by phenylethyl-NCS at 120–200 nM, by phenylbutyl-NCS at 30–75 nM, and by phenylhexyl-NCS at 15–90 nM depending on the specific metabolites. The  $K_i$  values for the inhibition of NNK metabolism by phenylhexyl-NCS were very low (10.9–16.8 nM) for various reactions (57).

Straightforward results were obtained in experiments in which 100–300  $\mu$ mol daily i.g. doses of allyl-NCS were given to male outbred rats for 3 days. The hepatic aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase, and aniline *p*-hydroxylase activities were all decreased in a dose-dependent manner to as low as 31–46% of control values (48).

Although both  $\alpha$ - and  $\beta$ -naphthyl-NCS block neoplasia in the liver (see above), dietary administration of these compounds to male F344 rats for 2–4 weeks had opposite effects on cytochrome P-450 content and monooxygenase activities of liver microsomes. Whereas the  $\alpha$ -naphthyl-NCS depressed total P-450 content, as well as ethoxycoumarin *O*-deethylase and benzphetamine *O*-demethylase activities, the  $\beta$  isomer had the opposite effects (27, 28). For instance, dietary  $\beta$ -naphthyl-NCS (5.4  $\mu$ mol/g diet for 28 days) raised microsomal cytochrome P-450 content, ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase 1.6-, 1.74-, and 2.2-fold, respectively (28).

In Hepa 1c1c7 murine hepatoma cells in culture, benzyl-NCS had no effect on aryl hydrocarbon hydroxylase activity (8).

Although the effects of isothiocyanates on Phase 1 enzymes appear to vary greatly with experimental conditions and the activity measured, the conclusion that these compounds depress the activation metabolism of two carcinogens, NNK and *N*-nitrosobenzylmethylamine, appears to be firmly established.

#### Regulation of Phase 2 Enzymes and Glutathione Levels by Isothiocyanates

In contrast to the complex effects of isothiocyanates on Phase 1 enzymes, the effect of these agents on Phase 2 enzymes of rodent tissues is quite straightforward. Table 2 summarizes the changes in specific activities of GST and QR in the cytosols of several organs of mice and rats treated with benzyl-NCS, phenethyl-NCS, allyl-NCS,  $\alpha$ -naphthyl-NCS,  $\beta$ -naphthyl-NCS, or sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane]. The compounds were given either in the diet (3–34  $\mu$ mol/g diet) for 5–28 days or by i.g. administration (5–100  $\mu$ mol in single or several daily doses). The organs examined included: liver; esophagus; forestomach; glandular stomach; small bowel; colon; lung; kidney; and bladder. Inductions of GST and QR, expressed as ratios of specific activities of tissues obtained from treated and control animals varied from 1.2 to 9.4 and were mostly in the 2–4-fold elevation range. Phase 2 enzyme induction, as repre-

Table 3 Effects of benzyl isothiocyanate on glutathione levels in female ICR/Ha mouse tissues

Tissue	Ratio of GSH concentrations (treated/control)	Ref.
Esophagus	1.63–1.75	Sporn's <i>et al.</i> (49)
Forestomach	0.77	Sporn's and Wattenberg (38)
Small bowel	1.64–1.66	Sporn's <i>et al.</i> (49)

Table 4 Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by isothiocyanates<sup>a</sup>

Isothiocyanate	Concentration required to double QR ( $\mu$ M)
Sulforaphane [ $\text{CH}_3\text{SO}(\text{CH}_2)_4\text{NCS}$ ]	0.2
<i>N</i> -Hexyl-NCS	15
Benzyl-NCS	1.9
Cyclooctyl-NCS	10
Cyclohexyl-NCS	50

<sup>a</sup> From Prester *et al.* (55).

sented by elevations of GST and QR in various tissues, appears to be a constant property of a variety of isothiocyanates. Benzyl-NCS also raised GSH levels in esophagus and small bowel (but not forestomach) of mice by 63–75% (Table 3; Ref. 38).

In common with many chemically unrelated chemoprotective compounds, the administration of isothiocyanates to rodents evokes a generalized "electrophile counterattack" response, characterized by the induction of Phase 2 enzymes and increases in tissue GSH levels (54, 55).

Much has been learned about the chemical structure and mechanism of induction of chemoprotective compounds by the measurement of QR activity in Hepa 1c1c7 murine hepatoma cells in culture (7), and especially in cells grown in 96-well microtiter plates (56). Elevation of QR in this cell-line accurately predicts induction of both QR and GST in animal tissues. This system played a critical role in the identification of sulforaphane as the major Phase 2 enzyme inducer in SAGA broccoli (10). Extensive information is now available on the inducer potency of a wide variety of isothiocyanates in Hepa cells. Some illustrative inducer concentrations required in the assay system to double the specific activity of QR in Hepa 1c1c7 murine hepatoma cells are given in Table 4.

In an effort to understand the basis for the high inducer potency of sulforaphane, a large number of bifunctional isothiocyanates were recently synthesized and evaluated as inducers (58). It was found that among these analogues, the methylsulfinyl group ( $\text{CH}_3\text{SO}-$ ) of sulforaphane could be replaced by either  $\text{CH}_3\text{CO}-$  or  $\text{CH}_3\text{SO}_2-$  groups without significant effect on inducer potency. For optimal potency, these functional groups had to be separated from the  $-\text{NCS}$  function by 3 or 4 carbon atoms. In some of the analogues, the link separating the two functions was flexible whereas in others it was relatively rigid. Certain bifunctional norbornyl-NCS analogues were potent inducers of QR in murine hepatoma cells and inducers of QR and GST in mouse tissues.

Since there is much persuasive evidence that induction of Phase 2 enzymes plays a major role in the chemoprotective effects of many different classes of compounds (9), it appears very likely that isothiocyanates also exert at least a part of their protective actions through this mechanism. Isothiocyanates are potent electrophiles like most other inducers of Phase 2 enzymes (7, 54, 55). Isothiocyanates resemble other monofunctional enzyme inducers in that they stimulate transcription of Phase 2 enzymes via a common AP-1-like enhancer element present in the upstream regulatory regions of certain GST and QR genes (54, 55).

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## THE ROLE OF ENZYME INDUCTION IN PROTECTION AGAINST CARCINOGENESIS\*

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### I. INTRODUCTION AND BACKGROUND

The notion that protection against the neoplastic effects of carcinogens can be achieved by administration of chemical agents is by no means new. Twenty-five years ago, in a review entitled "Chemo-prophylaxis of Carcinogenesis," Wattenberg<sup>1</sup> recorded experiments going back to 1929, in which chemical induction of tumors in rodents had been blocked by a variety of chemicals, including polycyclic aromatic hydrocarbons, phenothiazines, actinomycin D,  $\alpha,\beta$ -unsaturated dicarboxylic acids and anhydrides, dichlorodiethyl sulfide (mustard gas), metal ions, 2,3-dimercaptopropanol, 4-nitroquinoline *N*-oxide, and others. Although mouse skin was the experimental model for most of these studies, protection was also observed against systemic tumor formation in liver, mammary gland, and connective tissue, thereby indicating that protection did not arise solely from a local interaction between carcinogen and protector. One striking feature of these observations was that the protective agents were often carcinogens themselves and that under appropriate experimental conditions established carcinogens could act as protectors. Indeed, prior treatment with low doses of a carcinogen protected against higher doses of the same carcinogen.<sup>2</sup>

The striking finding of Richardson and Cunningham<sup>3</sup> that small doses of 3-methylcholanthrene (a potent carcinogen) provided substantial protection against the generation of liver tumors by the azo dye butter yellow (3'-methyl-*N,N*-dimethylaminoazobenzene) was followed many years later by the demonstration of the reverse phenomenon: protection against hydrocarbon (7,12-dimethylbenz(a)anthracene)-induced mammary tumor formation in rats by small doses of the azo dye, Sudan III.<sup>4</sup>

This solicited summary of work from our laboratory on chemoprotection against cancer is based on several recent reviews that are referenced in the bibliography. A similar chapter will appear as part of the Proceedings of a Workshop on "Cellular and Molecular Targets for Chemoprevention," sponsored by the National Cancer Institute and held in Charleston, South Carolina, on March 16-19, 1991.

Insight into the mechanisms responsible for these phenomena was provided by James and Elizabeth Miller and their coworkers,<sup>5,6</sup> who in the course of their analyses of the biochemical basis of chemical carcinogenesis attributed the protective effects to the greatly enhanced rates of oxidative and reductive detoxication of the carcinogenic azo dye (reduction of the azo linkage, ring hydroxylation, and *N*-demethylation) resulting from the induction of enzymes of xenobiotic metabolism. The responsible enzymes are now known as cytochromes P-450 (Phase I enzymes).<sup>7</sup> In retrospect this explanation is probably incomplete because enhancement of metabolic conjugation by Phase II enzymes (see below) undoubtedly also played a role in the protective phenomenon.

Carcinogenesis is a complex and protracted multistage process. Until recently, reduction or avoidance of exposure to carcinogens and early diagnosis were the only hopes of reducing the incidence of cancer or achieving better therapeutic results. It is now evident that the events that occur between exposure to carcinogens and the ultimate development of overt malignancy offer many opportunities for blocking or even reversing the neoplastic process. Our laboratory has focused on developing strategies for protecting cells against the effects of carcinogens by enhancing their metabolic inactivation, thereby promoting their elimination and reducing the opportunities for damaging interactions with cellular macromolecules.<sup>8,9</sup> Several considerations make this an especially appealing target for achieving chemoprotection: (a) protection is implemented at a very early step of the carcinogenic process, before damage to DNA occurs; (b) the enzymology of the metabolic reactions involved in carcinogen activation and inactivation is reasonably well understood; (c) many inducers of enzymes of carcinogen metabolism have been identified and the structures of others can now be predicted; and (d) a number of inducers of detoxication enzymes are relatively nontoxic; some occur naturally in living matter, and others are already minor components of the human diet.

## II. ENZYME INDUCTION AND CHEMOPROTECTION

### A. METABOLISM OF CARCINOGENS

The critical importance of metabolic activation of chemical carcinogens to their ultimate reactive forms was uncovered by the Millers and their colleagues<sup>10</sup> to whom we owe the central dogma of carcinogenesis: that ultimate carcinogens are electrophilic species that enter into damaging interactions with nucleophilic centers on the bases of DNA and thereby participate in carcinogenesis. These metabolic activations are generally promoted by one or more members of the family of inducible cytochromes P-450 (designated Phase I enzymes), which are membrane-bound hemoproteins that carry out mainly oxidations and reductions. Many (perhaps the majority) of the metabolic reactions catalyzed by cytochromes P-450 do not, however, lead to metabolic activations but to the formation of more water-soluble, nonelectrophilic detoxication products. Prominent among enzymes responsible for activation are cytochrome P-450IA1, involved in conversion of benzo(a)pyrene and other polycyclic aromatics to highly carcinogenic diol epoxides, and cytochrome P-450IA2, which participates in the enhancement of the carcinogenic functions of aromatic amines through their *N*-hydroxylation. Both of these cytochromes P-450 are inducible by a mechanism that requires the participation of the cytosolic *Ah* (Aryl hydrocarbon) receptor. The liganded *Ah* receptor is transported to the nucleus where it binds and activates XRE (Xenobiotic Responsive



An obvious corollary of this conclusion was that the balance between Phase I and Phase II enzymes could exercise critical control over the fate of carcinogens and that shifting this balance toward Phase II enzyme predominance could result in protection against electrophile toxicity and carcinogenesis. Subsequent observations from many laboratories have fully corroborated these suggestions.

#### B. MODULATION OF ENZYMES OF CARCINOGEN METABOLISM AND CONTROL OF CARCINOGENESIS

Since Wattenberg's 1966 review<sup>1</sup> of chemoprotectors, the list of compounds capable of protecting animals against the toxic and neoplastic effects of carcinogens has grown substantially, both in number and chemical diversity.<sup>22,23</sup> Chemoprotectors include not only small doses of polycyclic aromatics (some of which are themselves carcinogens) and azo dyes but also phenolic antioxidants such as BHA and BHT, coumarins, flavonoids, organic isothiocyanates, thiocarbamates (for example, disulfiram, diethyldithiocarbamate, bisethylxanthogen), 1,2-dithiole-3-thiones, indoles, and cinnamates.<sup>22,23</sup> It was difficult to understand how administration of such a bewildering variety of compounds could protect against carcinogenesis. Yet many experiments showed that these chemoprotectors were endowed with a singular and universal property: they were inducers of enzymes of xenobiotic metabolism in many tissues *in vivo* and in a variety of cells in culture. Many lines of evidence suggested that the ability to enhance the metabolic inactivation of carcinogens was largely responsible for the chemoprotective properties of these agents.<sup>8</sup>

More detailed analysis of the enzyme induction patterns evoked by these chemoprotectors led to the recognition that they fell into two quite distinct families, which we have designated *monofunctional* and *bifunctional* inducers.<sup>24,25</sup> Monofunctional inducers elevate the specific activities of Phase II enzymes without producing significant effects on the activities of cytochromes P-450 that are under the regulation of the *Ah* receptor. By contrast, bifunctional inducers are generally large planar aromatic compounds that bind to the *Ah* receptor and elevate the activities of both Phase I and Phase II enzymes. Because elevation of Phase II enzymes appears to be a sufficient condition for achieving protection against carcinogenic electrophiles, and Phase I enzymes can participate in carcinogen activation, chemoprotective strategies should ideally be implemented with monofunctional rather than bifunctional inducers.

#### C. EXPERIMENTAL SYSTEMS FOR THE STUDY OF MONOFUNCTIONAL INDUCERS

Because of the compelling evidence that Phase II enzyme induction is associated with a chemoprotected state (reduced susceptibility to the toxic and neoplastic effects of chemical carcinogens), we have developed simple experimental systems for identifying novel monofunctional inducers, for measuring their potencies, and for elucidating the molecular events responsible for induction. NAD(P)H:quinone oxidoreductase is a widely distributed FAD-containing flavoprotein that protects cells against the toxicities of quinones.<sup>14</sup> It is coordinately induced with other Phase II enzymes in most tissues. The murine hepatoma cell line designated Hepa 1c1c7 contains inducible quinone reductase that is easily measurable. This cell line therefore provides a reliable system for detecting and for measuring potency of monofunctional as well as bifunctional inducers.<sup>26,27</sup> The utility of this system has been greatly extended by growing Hepa 1c1c7 cells in microtiter plate wells and measuring

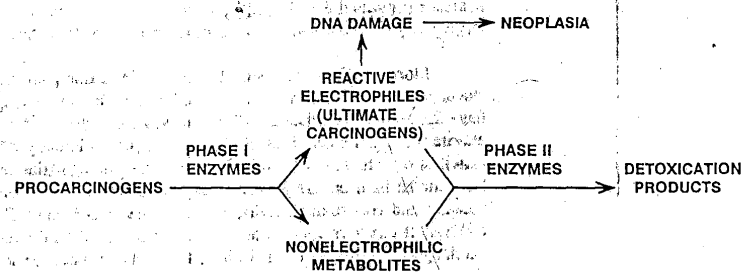
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Elements) in the upstream regions of these cytochrome P-450 genes.<sup>11</sup> Strong evidence for the importance of the *Ah* receptor in carcinogenesis is provided by the greatly reduced susceptibility to carcinogens of inbred mouse strains that lack or have low levels of this receptor.<sup>12,13</sup>

Protection of cells against the very reactive electrophilic metabolites of carcinogens and other xenobiotics is provided by the high intracellular levels of glutathione, which acts as a noncritical nucleophile scavenger to inactivate these toxic substances. Important defenses against electrophile toxicity are also provided by families of Phase II enzymes that inactivate these electrophiles by conjugation with glutathione (glutathione *S*-transferases), or with glucuronic acid (UDP-glucuronosyltransferases), or by hydrolysis. Another important detoxication enzyme is NAD(P)H:quinone reductase (quinone reductase), which catalyzes the obligatory two-electron reductions of quinones and thereby shields cells against the damaging electrophilicity of quinones and their ability to generate oxidative stress by oxidative cycling.<sup>14</sup> Phase II enzymes also conjugate and otherwise modify the nonelectrophilic products of Phase I enzyme action.

A simplified general scheme for the role of Phase I and Phase II enzymes in carcinogen metabolism is shown below:



The discovery by Wattenberg<sup>15,16</sup> that the phenolic antioxidant food additives, BHA [2(3)-*tert*-butyl-4-hydroxyanisole] and BHT (2,6-di-*tert*-butyl-4-hydroxytoluene), protected rodents against the toxic and neoplastic effects of a variety of carcinogens provided an important impetus to the development of the field of chemoprotection. Because these phenolic antioxidants were already present in the human diet, and clearly could not be very toxic, the prospects that other nontoxic chemoprotectors would be identified seemed much brighter.

Our analysis of the mechanism of the protective effects of these agents led to the startling observation that administration of phenolic antioxidants (and as subsequently shown, a wide variety of other chemoprotectors) produced marked elevations of glutathione transferases and other Phase II enzymes in many tissues without major effects on cytochromes P-450 (Phase I enzymes).<sup>17-21</sup> These findings suggested strongly that elevation of Phase II enzymes was a critical (and sufficient) condition for achieving chemoprotection.

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directly the specific activities of quinone reductase by means of a computer-coupled microtiter plate reader.<sup>28</sup> Quinone reductase is induced in these cells by all of the chemoprotectors shown in Figure 1.

#### D. IDENTITY OF CHEMICAL SIGNAL RESPONSIBLE FOR MONOFUNCTIONAL INDUCTION

Monofunctional inducers appear to act independently of any conventional receptor and superficially reveal few, if any, common structural features. Nevertheless a systematic study of the relation of structure to inducer potency led to the conclusion that all monofunctional inducers contain (or acquire by metabolism) electrophilic reaction centers.<sup>9,29</sup> This conclusion was reached in the following manner. When the inducer potencies of a series of alkyl ethers of 1,4-diphenols (designed as analogues of BHA) were examined, unsubstituted *tert*-butylhydroquinone was the most potent inducer and was active in tissues in which the ethers were only feebly active.<sup>30</sup> When these experiments were extended to include not only 1,4-diphenols but also 1,2-diphenols (catechols) and 1,3-diphenols (resorcinols), it was consistently found that only 1,2-diphenols and 1,4-diphenols were inducers, whereas 1,3-diphenols were completely inactive.<sup>27,31</sup> The appropriate orientation of the phenolic hydroxyl groups was absolutely critical, whereas the presence or absence of other ring substituents had only minor effects on inducer potency. Similarly, among phenylenediamines, the 1,2- and 1,4-isomers were inducers, but the 1,3-phenylenediamine was not. Since in these two series of compounds the 1,2- and 1,4-isomers are readily oxidized to quinones or quinoneimines whereas the 1,3-isomers cannot undergo such oxidations, we concluded that oxidative lability was an essential requirement for inducer activity.<sup>27</sup> Nevertheless, these experiments did not disclose whether the oxidative process (and for instance the attendant generation of reactive oxygen species) was responsible for the induction of Phase II enzymes, or whether the quinones or quinoneimines themselves provided the inductive signals.

Dissection of the structure of coumarin (an inducer lactone) and related compounds revealed that only the  $\alpha,\beta$ -unsaturated lactone moiety was required for inducer activity, and that indeed  $\alpha,\beta$ -unsaturated ketones were even more potent inducers.<sup>29</sup> These findings suggested that the critical structure responsible for inducer activity was the electrophilic olefin, which was activated by conjugation to a carbonyl or another electron-withdrawing group. The reactivity of such olefins is the basis for the well-known Michael reaction of electrophilic olefins with nucleophiles. This finding explained the inducer activity of oxidizable diphenols and phenylenediamines because their quinone and quinoneimine oxidation products, respectively, are excellent Michael reaction acceptors.

These generalizations not only explained the activities of many known inducers but also led to the prediction of inducer activity among acrylates, crotonates, cinnamates, fumarates, maleates, vinyl ketones, vinyl sulfones, and others.<sup>9,29,32,33</sup> Furthermore, the inducer potency of these compounds generally correlated well with their reactivity as Michael reaction acceptors. For instance, nitro-olefins such as 1-nitro-1-cyclohexene were very potent inducers, as expected on the basis of the highly potent electron-withdrawing effect of the nitro group.

In light of the natural occurrence of fumarates in animal tissues and the generally recognized low toxicity of fumarate, which is used as a food additive, we have examined the

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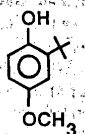
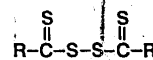
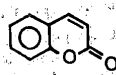
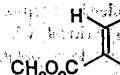
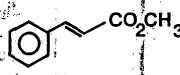
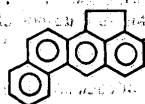
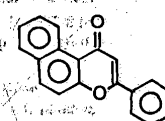
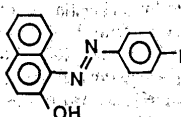
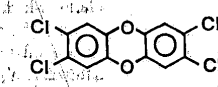
**EXAMPLES OF MONOFUNCTIONAL INDUCERS****BHA****BENZYL ISOTHIOCYANATE****1,2-DITHIOLE****THIO-CARBAMATES****COUMARIN****DIMETHYL FUMARATE****METHYL CINNAMATE****EXAMPLES OF BIFUNCTIONAL INDUCERS****3-METHYL-CHOLANTHRENE** **$\beta$ -NAPHTHOFLAVONE****SUDAN III****2,3,7,8-TETRACHLORO-DIBENZO-p-DIOXIN (TCDD)**

FIGURE 1. Examples of monofunctional inducers that elevate Phase II enzymes selectively and bifunctional inducers that raise Phase I and Phase II enzymes. For dithiocarbamates: disulfiram ( $R = (C_2H_5)_2N$ ); bisethyloxanthogen ( $R = C_2H_5O-$ ).



## IV. ACKNOWLEDGMENTS

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inducer activity of dimethyl fumarate in rodent tissues. Dietary administration of this compound to mice and rats raised the specific activities of quinone reductase and glutathione transferase in many tissues two- to elevenfold.<sup>32</sup> These findings have encouraged us to suggest that dimethyl fumarate be examined for its protective activity against carcinogenesis. This suggestion gains validity from the finding that fumaric acid itself is a plant product and is endowed with a variety of pharmacological properties, including the ability to block chemical carcinogenesis and other forms of electrophile toxicity (see ref. 32 and references therein).

#### E. INDUCERS ARE SUBSTRATES FOR GLUTATHIONE TRANSFERASES

The striking similarity of the requirements for inducer activity and for substrates of glutathione transferases became immediately apparent as the structure/activity relationship was developed.<sup>33</sup> Thus in an extensive survey of glutathione transferase substrates, Chasseaud<sup>34</sup> described many of the very same compounds that were subsequently shown to be inducers of Phase II enzymes. A systematic survey of the ability of a broad range of glutathione transferase substrates to induce quinone reductase activity in Hepa 1c1c7 cells revealed virtual identity of structural requirements. Thus, for instance, the following transferase substrates were all inducers: 1-chloro-2,4-dinitrobenzene, methyl iodide, 1,4-dichloro-2-nitrobenzene, ethacrynic acid, cumene hydroperoxide, and *tert*-butyl hydroperoxide.<sup>35</sup> However, it is not clear whether this correlation of requirements for inducer and substrate activity occurs because both processes depend on the presence of electrophilic centers, or whether glutathione transferases participate in the transmission of the electrophilic inducer signal to activate the enhanced transcription of Phase II enzymes.

### III. CONCLUSIONS AND SUMMARY

The susceptibility of biological systems to carcinogens is controlled at least in part by the balance between Phase I enzyme systems (cytochromes P-450), which activate carcinogens to their ultimate and highly reactive electrophilic forms, and Phase II enzymes (for example, glutathione transferases, epoxide hydrolases, and NAD(P)H:quinone reductase), which convert these carcinogens to less toxic products. Both Phase I and Phase II enzymes are induced by a variety of chemical agents. Such inducers fall into two families: bifunctional inducers (mostly large planar aromatics) that induce both Phase I and Phase II enzymes, and monofunctional inducers that elevate Phase II enzymes selectively. Protection can be achieved by raising the levels of Phase II enzymes only, and because Phase I enzymes are involved in the activation of carcinogens, practical strategies for chemoprotection ideally should concentrate on monofunctional inducers. Monofunctional inducers contain, or acquire by metabolism, electrophilic centers, and many are Michael reaction acceptors. It is therefore quite easy to design or to identify monofunctional inducers, and many inducers of this type are already present in the human diet. The feasibility of modifying the human diet to increase the consumption of foods containing monofunctional inducers needs consideration as a strategy for chemoprotection.

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Analysis of the anticarcinogenic mechanisms of BHA identified the important protective role of the electrophile counterattack response. It was found that administration of BHA to rodents increased the specific activities of glutathione transferases, UDP-glucuronosyltransferases, epoxide hydrolase, NAD(P)H:quinone reductase, and other Phase 2 enzymes in the liver and peripheral tissues of these animals, and also raised levels of reduced GSH (1-4). The activities of Phase 1 enzymes that are regulated by the *Ah* receptor (i.e. cytochromes P-450 1A1 and 1A2) were largely unaffected by BHA treatment (5). BHA was thus classified as a *monofunctional* inducer, in contrast to *bifunctional* inducers (e.g., polycyclic aromatics, azo dyes, flavonoids); which elevate both Phase 1 and Phase 2 enzymes (5). These findings led to the formulation of the now widely held view that elevation of Phase 2 enzymes is mainly responsible for the protective effects of BHA against neoplasia and other forms of electrophile toxicity (7). Many lines of evidence attest to the validity of this conclusion. Indeed several novel anticarcinogens have been identified on the basis of their ability to evoke Phase 2 enzyme inductions (8-10).

This paper describes the biological scope of the electrophile counterattack response; the chemistry of the inducers, and the molecular regulation of the response (6).

#### BIOLOGICAL SCOPE OF THE ELECTROPHILE COUNTERATTACK RESPONSE

*Induction of phase 2 enzymes.* The earliest and up to now most complete information on the biochemistry of the electrophile counterattack response has been obtained on animals and cells treated with BHA or *tert*-butylhydroquinone, which is presumed to be its active metabolite. Feeding of 0.5-1.0% BHA in the diet to mice or rats for 5-7 days profoundly reduced the conversion of benzo(a)pyrene to mutagenic metabolites (1). The livers and peripheral tissues of these animals showed large increases in the specific activities of GSTs (2, 4) and their cognate mRNAs (11-13). Indeed, these normally rather abundant enzymes increased even further and became the most prominent proteins on two-dimensional electrophoresis of mouse liver cytosols (11). Subsequent studies revealed that the elevated GSTs belonged to the  $\alpha$ ,  $\mu$ , and  $\pi$  families and that the inductions showed considerable tissue specificity (12, 14-16).

Further studies demonstrated that the capability of chemoprotective agents to elevate enzymes of xenobiotic metabolism was not limited to BHA and was a widespread property of many chemoprotectors. Moreover, the variety of enzymes induced was diverse (7), and included UDP-glucuronosyltransferases, NAD(P)H:(quinone acceptor) oxidoreductase, epoxide hydrolase and a number of additional Phase 2 enzymes (3, 4, 17, 18). A comprehensive review of the protective effects of BHA and

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## THE ELECTROPHILE COUNTERATTACK RESPONSE: PROTECTION AGAINST NEOPLASIA AND TOXICITY

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### INTRODUCTION

Neoplasia is one important manifestation of genotoxicity, and commonly arises from the interaction of exogenous or endogenously-generated electrophiles with critical nucleophilic centers of DNA. Oxidative cycling, which generates reactive oxygen species (superoxide, peroxide, and hydroxyl radicals), also contributes to DNA damage and to the development of malignancy. Both electrophiles and reactive oxygen species deplete intracellular glutathione and thereby aggravate these toxicities. Hence, it is not surprising that cells have developed elaborate mechanisms to counteract these dangers.

Exposure of animals or their cells in culture to low but tolerated concentrations of electrophiles evokes a series of characteristic and wide-ranging metabolic responses including the coordinated induction (by enhanced transcription) of a number of Phase 2\* enzymes that detoxify electrophiles, and the elevation of intracellular levels of reduced GSH (by accelerated synthesis). These responses reduce the susceptibility of cells to higher, potentially hazardous, or even lethal, concentrations of the same or other toxic electrophiles. We suggest that this constellation of protective metabolic responses be designated as: "*Electrophile Counterattack*".

Abbreviations and definitions: Ah receptor, Aryl hydrocarbon receptor; BHA, 2(3)-tert-butyl-4-hydroxyanisole; CD, concentration of a compound that doubles the specific activity of quinone reductase in Hepa 1c1c7 cells under specified conditions; GSH, reduced glutathione; QR, quinone reductase, NAD(P)H:(quinone acceptor) oxidoreductase (EC 1.6.99.2); GST, glutathione transferase (EC 2.5.1.18).

\*Enzymes involved in the metabolism of xenobiotics have been classified into two broad categories. Phase 1 enzymes (principally cytochromes P-450) functionalize compounds largely by oxidative or reductive reactions, whereas Phase 2 enzymes carry out the conjugations of such functionalized compounds with endogenous ligands (e.g., glutathione and glucuronic acid). Quinone reductase is classified as a Phase 2 enzyme because it serves protective functions, is induced coordinately with other Phase 2 enzymes, and is regulated by enhancer elements that are similar to those controlling Phase 2 enzymes (6).

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other synthetic antioxidants, their biological effects, and enzyme induction patterns has been provided by R. Kahl (19).

Typical changes in the activities of Phase 2 enzymes in the livers of mice treated with BHA are shown in Figure 1A. From 5- to 10-fold increases in specific activities of GST, QR, epoxide hydrolase, and UDP-glucuronosyltransferases were observed. In contrast, several cytochrome P-450-related functions (total cytochrome P-450 hemoprotein, aniline hydroxylase, and aminopyrine demethylase) were unaffected or even depressed by such treatment. Based on the marked changes in Phase 2 enzymes in response to BHA administration, it was suggested that

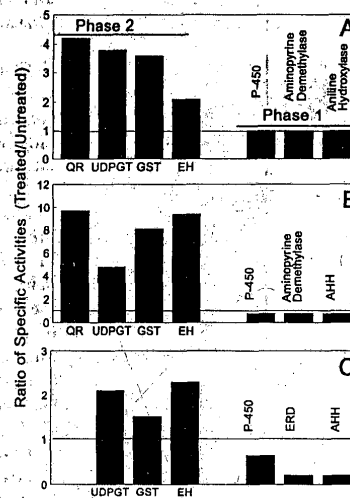


FIG. 1: Effect of monofunctional inducers on specific activities of Phase 1 and Phase 2 enzymes in rodent livers, expressed as the ratios of specific activities of cytosols or microsomes of treated to untreated animals. (A) Feeding of BHA to female CD-1 mice (from Refs (2-4; 17; 18)). (B) Feeding of 1,2-dithiole-3-thione to male F 344 rats (from Ref. (21)). (C) Intraperitoneal administration of bromobenzene to male Sprague-Dawley rats (from Ref. (22)). Note the large increases in the specific activities of glutathione transferases (GST), quinone reductase (QR), UDP-glucuronosyltransferases (UDPGT), and epoxide hydrolase (EH). Also note the lack of increase (or possibly decline) in the total cytochrome P-450 content (P-450), and aminopyrine demethylase, aniline hydroxylase, aryl hydrocarbon hydroxylase (AHH), and ethoxyresorufin *O*-deethylase (ERD) activities.

BHA might block chemical carcinogenesis by enhancing the detoxication capacity of tissues for the ultimate carcinogenic electrophiles, and that the balance between Phase 1 and Phase 2 enzymes played a major role in determining the outcome of the interaction of cells with carcinogens (2, 7, 20). A wealth of evidence from many laboratories supports the validity of these suggestions (20).

The wide range of chemical agents that evoke the electrophile counter-attack is exemplified in this paper by the effects on liver enzyme profiles of two other xenobiotics: 1,2-dithiole-3-thione, which belongs to a class of compounds that are recognized chemoprotectors (Fig. 1B) (21), and bromobenzene (Fig. 1C) (22), which has not to our knowledge been tested as a chemoprotector. Thus administration of two agents that appeared to be chemically unrelated to each other and to BHA evokes similar and characteristic alterations in the profiles of xenobiotic-metabolizing enzymes.

*Elevations of cellular glutathione levels.* It has been recognized for many years that the normally high intracellular GSH levels protect cells against a wide variety of toxic chemicals including electrophiles and reactive oxygen species. These protective processes involve both enzymatic (GSTs, glutathione peroxidase) and nonenzymatic reactions (23, 24). Administration of BHA and other substances that elevate Phase 2 enzymes also raised the levels of GSH in the liver and other mouse tissues (1, 4). Similarly, the feeding of low concentrations of 1,2-dithiole-3-thione raised rat hepatic GSH levels 3-fold (21). With the recognition that many chemoprotectors are electrophiles, these observations initially seemed paradoxical, because electrophiles (e.g., diethyl maleate) have been used extensively to deplete the intracellular GSH levels (25). However, depletion of GSH requires high (i.e. stoichiometric or millimolar) concentrations of such electrophiles. Consequently, the findings of Bannai (26-28) and those of Deneke *et al.* (29) that exposure of fibroblasts, endothelial cells, or peritoneal macrophages to micromolar concentrations of diethyl maleate or other electrophiles resulted in substantial elevations of GSH levels are particularly pertinent. The electrophile concentrations required to raise GSH levels are similar to those that induce Phase 2 enzymes. The increases in GSH have been shown to involve enhanced synthesis of a highly specific, sodium-independent, transport system for anionic cystine (in exchange for glutamate). The intracellular concentration of cysteine (derived from imported cystine) is rate-limiting for GSH synthesis. The electrophiles that were shown by Bannai (26) to enhance cystine transport and to raise GSH levels include cyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-one, ethacrynate, maleate, fumarate, cinna-



their phenylenediamine analogs) undergo facile oxidations to quinones, whereas 1,3-diphenols and 1,3-phenylenediamines cannot participate in such oxidations. Although these experiments did not establish whether the oxidation products or the oxidation processes (potentially involving multiple one- and two-electron oxido-reductions and the generation of reactive oxygen species) were the inductive signal, subsequent work showing the important role of Michael reaction acceptors as inducers strongly suggested that the quinone (or quinoneimine) oxidation products were the ultimate inducers, since these products are strongly electrophilic (30, 31).

(2) *Michael reaction acceptors.* The recognition that many inducers were compounds containing olefins or acetylenes conjugated to electron-withdrawing groups was a major advance in understanding the nature of the inducer signal (30, 31). Such inducers include olefins conjugated with aldehydes, ketones (including thioketones and quinones), esters (e.g., acrylates, crotonates and cinnamates, as well as lactones), nitriles, and nitro groups, i.e. Michael reaction acceptors. The potencies of these compounds as inducers were generally related to the avidities of the electron-withdrawing functions and paralleled the efficiencies of the compounds as Michael reaction acceptors. The concentration dependence of the induction of QR in murine hepatoma cells by a number of selected Michael reaction acceptors is shown in Figure 2.

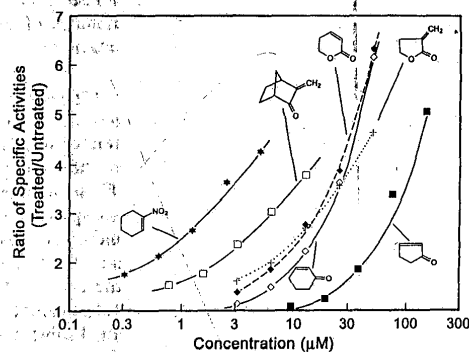


FIG. 2. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of Michael reaction acceptors. The increases in the specific activities of QR are expressed as the ratios of values obtained from treated to untreated cells. Assays were performed in microtiter plates by slight modifications (6, 30) of the method of Prochaska and Santamaria (32). The inducers shown are identified as Nos 1-6 in Figure 7.

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mate, 1,2-epoxyethylbenzene, 1,2-ethoxy-3-(*p*-nitrophenoxy)propane, *p*-nitrobenzyl chloride, and bromosulphophthalein. It is remarkable that the same or very similar electrophiles are also inducers of QR in murine hepatoma cells (30, 31). In connection with findings to be discussed below, it is also of interest that stimuli commonly associated with stress-protein synthesis, such as sodium arsenite, cadmium chloride, and hyperoxia also enhanced cystine transport and GSH levels (28).

It may be concluded therefore that low concentrations of electrophiles, or of compounds that are converted to electrophiles by metabolism (e.g., BHA), induce a variety of Phase 2 enzymes, and elevate GSH levels through enhanced precursor transport.

## CHEMISTRY OF INDUCERS

Structure-activity studies have provided considerable insight into the chemical requirements for inducer activity and the nature of the chemical signal that evokes the elevations of Phase 2 enzymes and GSH levels. Most of the quantitative information on inducer potency has been obtained by measuring QR levels in Hepa 1c1c7 murine hepatoma cells grown and exposed to these inducers in 96-well plates (30, 32). We have established that the response of QR to compounds in this cell culture system is a reliable predictor of inducer activity for QR, GST, and other Phase 2 enzymes in the liver and peripheral tissues of rodents (5; 33, 34).

The growing number and variety of compounds known to evoke these Phase 2 enzyme inductions include at least seven chemically distinct families of monofunctional inducers, i.e. selective inducers of Phase 2 enzymes (6, 30, 31).

(1) *Diphenols, phenylenediamines, and quinones.* From studies of the induction of QR and GST in murine liver by a series of 1,4-dialkyl phenols related to BHA, it was concluded that induction depended on conversion of these compounds to 1,4-diphenols such as *tert*-butylhydroquinone (35). These findings raised the issue whether the 1,4-disposition of the hydroxyl groups was essential for inducer activity. Extensive comparisons of the inducer potencies of 1,2-diphenols (catechols), 1,3-diphenols (resorcinols), and 1,4-diphenols (hydroquinones) demonstrated conclusively that only the 1,2- and 1,4-diphenols were inducers of Phase 2 enzymes, whereas the 1,3-diphenols were always inactive (33, 36). The presence or absence of alkyl substituents on the aromatic nuclei played a relatively minor role in determining inductive potency. Analogous experiments with phenylenediamines demonstrated similar structural requirements, i.e. only the 1,2- and 1,4-phenylenediamines were inducers, but the 1,3-phenylenediamines were not. These findings led to the conclusion that oxidative lability was essential for inducer activity since catechols and hydroquinones (and

(5) *Mercaptans*. Vicinal dimercaptans such as 2,3-dimercaptopropanol ( $CD = 13 \mu M$ ) and 1,2-ethanedithiol ( $CD = 34 \mu M$ ) were reasonably potent inducers of QR in murine hepatoma cells (Table 1). This was an unexpected finding since most previously known inducers were electrophiles: *Meso*-2,3-dimercaptosuccinic acid ( $CD = 120 \mu M$ ) and its dimethyl ester ( $CD = 26 \mu M$ ) were also inducers. However, the monothiol 2-mercaptoethanol ( $CD = 220 \mu M$ ) was a much weaker inducer, and non-vicinal dithiols such as 1,4-dithiothreitol and 1,4-dithioerythritol were inactive. The relatively high potency of the vicinal mercaptans as inducers is difficult to reconcile with the other structure-activity results. Dimercaptopropanol and dimercaptosuccinates are widely used as metal chelators in the treatment of heavy metal poisonings. These compounds also react with sulfhydryl groups. Whether the redox properties, the metal chelation properties, the ability to react with mercaptans, or some other property of dimercaptans is responsible for the induction of Phase 2 enzymes is currently unclear.

TABLE 1. INDUCTION OF QUINONE REDUCTASE (QR) IN HEPA 1c1c7 CELLS BY MERCAPTANS

Compound	Concentration required to double QR specific activity ( $\mu M$ )
2,3-Dimercaptopropanol (BAL)	$12.9 \pm 3.8^* (N = 12)$
1,2-Ethanedithiol	$33.6 \pm 9.8 (N = 6)$
1,2-Propanedithiol	20
2-Mercaptoethanol	220
<i>meso</i> -2,3-Dimercaptosuccinic acid	120
Dimethyl <i>meso</i> -2,3-Dimercaptosuccinate	26
Dithiothreitol	In†
Dithioerythritol	In
1,4-Butanedithiol	In

\*  $\pm$  S.D.

† Less than 20% increase at  $100 \mu M$ .

(6) *Arsenicals*. Trivalent arsenic derivatives are excellent inducers of QR in Hepa 1c1c7 cells (Fig. 4). The hydrophobic phenylarsine oxide ( $CD = 0.057 \mu M$ ) is one of the most potent inducers so far uncovered, and is considerably more potent than sodium arsenite ( $CD = 2.1 \mu M$ ). Sodium arsenate, a pentavalent arsenical, was much less active ( $CD = 12 \mu M$ ). The greater potency of the trivalent arsenicals correlates well with their much higher reactivity with vicinal (or closely-spaced) sulfhydryl groups, and the greater facility with which trivalent arsenicals enter cells (39). These findings are also consistent with the notion that pentavalent arsenic

(3) *Isothiocyanates* ( $R-N=C=S$ ). These compounds are widely distributed in plants and their seeds, where they are often present in the form of glucosinolates (thioglucoside, *N*-hydroxysulfate derivatives) and are accompanied by thioglucoside hydrolases (myrosinases) that convert these compounds to isothiocyanates,  $HSO_3^-$ , and glucose (37). The inducer potencies of isothiocyanates vary widely; only those possessing at least a single hydrogen on the  $\alpha$ -carbon atom are inducers (e.g., *tert*-butyl and phenyl isothiocyanates are inactive) (30). Sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane:  $CH_3-SO-(CH_2)_4-N=C=S$ ], isolated from broccoli and other crucifers, is a very potent inducer ( $CD = 0.21 \mu M$ ) (10). The responses of QR in murine hepatoma cells to sulforaphane and several other isothiocyanates are shown in Figure 3.

(4) *Hydroperoxides*. Cumene hydroperoxide ( $CD = 210 \mu M$ ) and *tert*-butyl hydroperoxide ( $CD = 140 \mu M$ ), as well as extremely high concentrations of hydrogen peroxide ( $CD = 560 \mu M$ ) are also inducers (6, 38). Whereas the hydroperoxides are substrates for GST, hydrogen peroxide is not thought to be a substrate for these transferases, although it is not clear that it has been tested at the high concentrations required to achieve inductions.

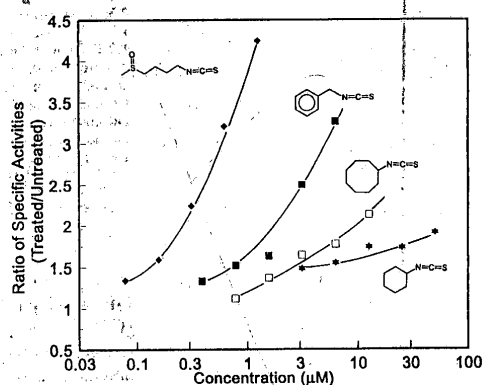


FIG. 3. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of isothiocyanates. The specific activities of QR are presented and were obtained as described in Fig. 2. The inducers are: sulforaphane ( $CD = 0.21 \mu M$ ), and benzyl ( $CD = 1.9 \mu M$ ), cyclooctyl ( $CD = 10 \mu M$ ), and cyclohexyl ( $CD = 50 \mu M$ ) isothiocyanates.



derivatives must undergo cellular reduction in order to exert biological (chemotherapeutic or toxic) activity (40). Trivalent arsenicals are classical sulfhydryl reagents that form covalent heterocyclic adducts with vicinal or adjacent sulfhydryl groups (see review in (41)). The potent induction of QR by such compounds suggests the critical presence of two neighboring sulfhydryl groups on the protein(s) that receive and transmit the inductive signal.

(7) *Heavy metals.* The potencies of  $\text{HgCl}_2$ ,  $\text{CdCl}_2$ , and  $\text{ZnCl}_2$  as inducers of QR in murine hepatoma cells correlated with their affinities for sulfhydryl groups (Fig. 5).  $\text{HgCl}_2$  ( $CD = 0.76 \mu\text{M}$ ) was much more potent than  $\text{CdCl}_2$  ( $CD = 10.5 \mu\text{M}$ ), whereas  $\text{ZnCl}_2$  ( $CD = 230 \mu\text{M}$ ) was only weakly active. Cadmium and mercury are soft electrophiles which are ideal reagents for thiol groups as these are soft nucleophiles. It may therefore be of special significance that phenylmercuric chloride ( $CD = 0.1 \mu\text{M}$ ) and *p*-chloromercuribenzoate ( $CD = 1.2 \mu\text{M}$ ) which were designed as sulfhydryl reagents are both potent inducers.

#### MOLECULAR REGULATION OF ELECTROPHILE COUNTERATTACK RESPONSE

Recently, deletion analyses of the 5'-upstream regulatory regions of two Phase 2 enzyme genes: the Ya GST gene (of mouse and rat liver) and the QR gene (of human and rat liver), have been carried out by transient gene expression assays with the use of chloramphenicol acetyltransferase (CAT). The upstream regions of the mouse and rat liver GST Ya genes contain very similar enhancer sequences located within a 41-bp region. These similar, but not identical, enhancer sequences have been designated the Electrophile Responsive Element (EpRE) in the mouse (42) and the Antioxidant Responsive Element (ARE) in the rat (43, 44) GST Ya genes, respectively. The 41-nucleotide DNA segment is located between bp -714 and -754 in the mouse, and -682 and -722 in the rat from the transcription start (Fig. 6). In the mouse, the critical DNA sequences responsive to the few monofunctional inducers tested appear to be the two tandem TGACAT/AT/AGC regions separated by a 6-bp sequence. These two regions have been likened to AP-1 sites, and evidence has been obtained for their functioning in this capacity (45). In the rat GST Ya gene, the single enhancer sequence has been deduced to have the consensus: 5'-puGTGACNNNGC (44). Similar enhancer sequences have also been identified in the upstream regulatory regions of the rat and human quinone reductase genes (45, 47). Only very few of the wide variety of inducers described above had been examined with these transient gene expression constructs. Consequently we recently undertook a comprehensive comparison of the potencies of

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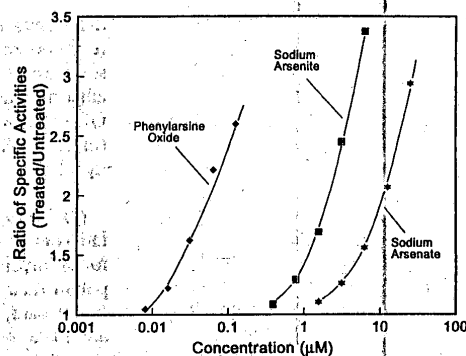


FIG. 4. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of arsenic derivatives. The specific activities of QR are presented and were obtained as described in Fig. 2. The arsenicals are: phenylarsine oxide (No. 26 in Fig. 7;  $CD = 0.057 \mu M$ ); sodium arsenite (No. 27;  $CD = 2.1 \mu M$ ), and sodium arsenate ( $CD = 12 \mu M$ ).

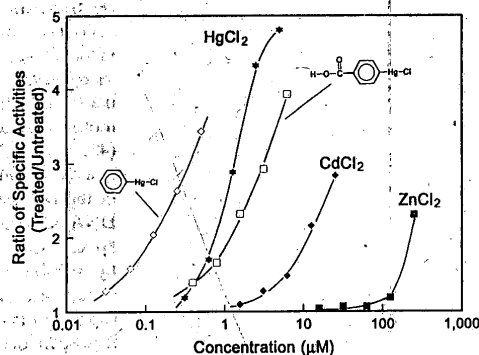


FIG. 5. Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of metal derivatives. The specific activities of QR are presented and were obtained as described in Figure 2. The inducers are: phenylmercuric chloride (No. 31 in Fig. 7;  $CD = 0.1 \mu M$ );  $HgCl_2$  ( $CD = 0.76 \mu M$ ); *p*-chloromercuribenzoic acid (No. 32;  $CD = 1.2 \mu M$ );  $CdCl_2$  ( $CD = 10.5 \mu M$ ); and  $ZnCl_2$  ( $CD = 230 \mu M$ ).

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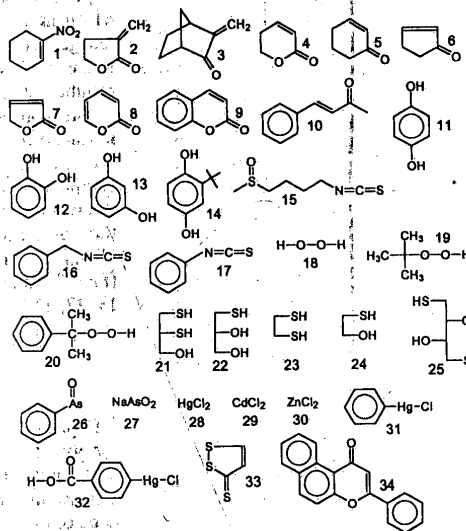
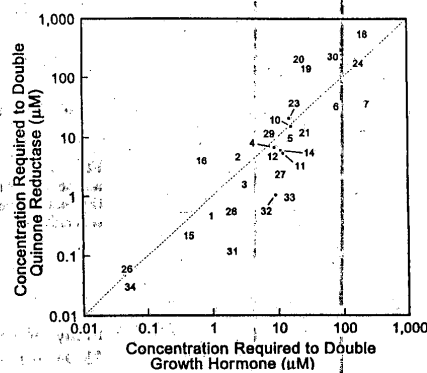
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FIG. 7. Caption opposite.



Posner. We thank Gale Doremus for help in preparing this manuscript and the illustrations.

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this cellular adaptation, which occurs in the liver and many peripheral tissues, be designated as the "Electrophile Counterattack" response. Seven families of highly diverse chemical agents that elicit this response include: oxidatively labile diphenols and quinones; Michael reaction acceptors (olefins conjugated to electron-withdrawing groups); isothiocyanates; organic hydroperoxides; vicinal dimercaptans; trivalent arsenicals; heavy metals ( $\text{HgCl}_2$ ,  $\text{CdCl}_2$ ) as well as mercury derivatives with high affinities for sulfhydryl groups; and 1,2-dithiole-3-thiones. An analysis of the molecular mechanisms of these enzyme inductions was carried out by transient expression in hepatoma cells of a plasmid containing a 41-bp enhancer element derived from the 5'-upstream region of the mouse glutathione transferase Ya gene, and the promoter region of this gene, linked to a human growth hormone reporter gene. The concentrations of 28 inducers (belonging to the seven chemical classes) required to double growth hormone production in this system spanned a range of four orders of magnitude and were closely and linearly correlated with the concentrations of the same compounds required to double the specific activity of quinone reductase in murine hepatoma cells. We therefore conclude that the regulation of these Phase 2 enzymes (and possibly also that of glutathione synthesis) by all of these inducers is mediated by the same enhancer element that contains AP-1-like sites. Similar enhancer sequences are present in the rat glutathione transferase Ya gene, and in the upstream regulatory regions of the quinone reductase genes of rat and human liver.

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FIG. 7. Comparison of potencies of 28 compounds as inducers of quinone reductase and stimulators of growth hormone production in a transient gene expression assay. A total of 34 compounds were tested both for their ability to induce QR in Hepa 1c1c7 cells and for their ability to increase transcription in Hep G2 cells of a reporter gene linked to the 41-bp EpRE element from the mouse GST Ya upstream region (6). Twenty-eight of the compounds tested were active in both assays. These potencies are plotted according to the concentration of each compound required to double the quinone reductase specific activity (ordinate) and the concentration of compound required to double the expression of the growth hormone reporter construct (abscissa). The numbers on the graph correspond to the structures shown below. Compound Nos 8, 9, 13, 17, 22, and 25 were inactive and are not included.



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